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Phospholipase D activation regulates endothelin-1 stimulation of phosphoinositide-specific phospholipase C in SK-N-MC cells

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Endothelin-1 (ET-1) is known to stimulate phospholipase C (PLC) activity in SK-N-MC human neuroblastoma/epithelioma cells: here we show that phospholipase D (PLD) is also stimulated. The generation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) by ET-1-stimulated PLC was attenuated by protein kinase C (PKC) activation and enhanced by PKC inhibition. An enhancement of ET-1-stimulated Ins(1,4,5)P_3 accumulation was also seen when the product of PLD activity was either diverted into phosphatidyl butanol in the presence of butanol, or phosphatidate phosphohydrolase (PPH) activity was inhibited by DL-propranolol. We conclude that there is an inhibitory, PKC-mediated, feedback loop in these cells which is dependent, in part, on the activation of PKC by product(s) of the PLD/PPH pathway. This provides a novel role for agonist-stimulated PLD activation.

Phospholipase D; Protein kinase C; Phospholipase C; Endothelin-1; Inositol 1,4,5-trisphosphate; SK-N-MC cell

1. INTRODUCTION

Inositol phospholipid hydrolysis by agonist-stimulated phosphoinositide-specific phospholipase C (PLC) has been shown to be modulated by protein kinase C (PKC) in many systems. For example, direct activation of PKC by phorbol esters has been shown to decrease, whilst PKC inhibition or down-regulation can facilitate, agonist-stimulated inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3) accumulation [1–4]. Such data are strongly suggestive of the existence of a short negative feedback loop by which PKC activation attenuates agonist-stimulated PLC activity.

In many cases of stimulation of PLC by receptors it has been reported that phospholipase D (PLD) activation also occurs. It seems very likely that PLD activation commonly occurs downstream of PLC: in a number of instances this has been shown to involve PKC [5–7]. The primary lipid product of PLD is phosphatidic acid (PtdOH) which may be converted to diacylglycerol by phosphatidate phosphohydrolase (PPH), and so the PLD route may serve as an alternative, agonist-activated pathway for stimulation of PKC. Despite various suggestions for a second messenger role for PtdOH itself, and intense interest in the PLD/PPH pathway, there is no established role for PLD in short-term cell signalling processes.

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Here we investigate signalling events in a human neuroblastoma/epithelioma-derived cell line (SK-N-MC) stimulated by endothelin-1 (ET-1) acting on ET_A receptors which are linked to PLC [8,9]. We show that the PLC response is profoundly attenuated by a short PKC inhibitory loop, and provide evidence that this feedback loop is activated by a product of agonist-stimulated PLD.

2. MATERIALS AND METHODS

2.1 *Materials*

ET-1 was purchased from the Peptide Research Institute (Osaka, Japan). Ro 31-8220 was a kind gift of Dr. G. Lawton, Roche Products Ltd. (Welwyn, UK). Cell culture supplies were from Gibco (Paisley, Scotland). Other chemicals were from Sigma or Fisons. $^{32}P_i$ was from Amersham and *myo*-[2-^{3}H]inositol and [^{3}H]Ins(1,4,5)P_3 were from NEN-DuPont.

2.2. *$^{32}P_i$ labelling and PLD assay*

The procedures for labelling cells with $^{32}P_i$ and subsequent analysis have been described and validated by Purkiss and Boarder [7]. Briefly, SK-N-MC cells (passage 55–60) were loaded with $^{32}P_i$ (0.25 MBq/ml) in 0.4 ml of a phosphate-free balanced salt solution for 24 h. The loading medium was replaced with 0.5 ml balanced salt solution, and after 10 min, the medium was again replaced by fresh medium containing ET-1 and butanol. Where appropriate additions of 12-*O*-tetradecanoyl-phorbol 13 acetate (TPA) and/or Ro 31-8220 were present throughout the incubation period. Incubations were terminated with methanol/$CHCl_3$. [^{32}P]Phosphatidylbutanol (PtdBut), which is a unique product of PLD and thus an index of PLD activity, was separated by thin layer chromatography.

2.3. *Measurement of total [^{3}H]inositol phosphates and Ins(1,4,5)P_3*

For assessment of [^{3}H]inositol phosphate ([^{3}H]InsP_x) production, cells were loaded for 48 h with [^{3}H]inositol (0.074 MBq/ml) in 0.5 ml Dulbecco's modified Eagle's medium with 1% non-essential amino acids. After washing, cell monolayers were preincubated for 10 min

with 10 mM LiCl, which was also present during the 30 min incubation period with ET-1. Where indicated additions of TPA, Ro 31-8220, butanol and DL-propranolol were made to both pre-incubation and incubation media. Incubations were terminated with cold acidified methanol/$CHCl_3$. [^{3}H]$InsP_x$ were separated by Dowex-1 (Cl^- form) ion exchange chromatography. For experiments where Ins(1,4,5)P_3 mass was measured, unlabelled cells were pre-incubated/incubated as described above except that LiCl was omitted and incubations were terminated by medium aspiration and addition of 0.5 M trichloroacetic acid. Samples were processed and Ins(1,4,5)P_3 determined as described previously [10].

3. RESULTS AND DISCUSSION

Preliminary experiments established that an ET-1-stimulated increase in [^{32}P]PtdBut accumulation could be demonstrated in SK-N-MC cells pre-labelled with $^{32}P_i$ and stimulated in the presence of 50 mM butanol. The effect of ET-1 (1–100 nM) was shown to be concentration-dependent, with a 288 ± 45% stimulation of [^{32}P]PtdBut accumulation being observed in response to 100 nM ET-1; time-course studies revealed a plateau in [^{32}P]PtdBut accumulation by 3–5 min. ET-1-stimulated [^{32}P]PtdBut accumulation (3 min incubations) was attenuated in the presence of the protein kinase C inhibitor Ro 31-8220; furthermore, addition of phorbol ester alone did not increase [^{32}P]PtdBut accumulation (e.g. control, 311 ± 38; + ET-1 (100 nM), 736 ± 87; + ET-1 + Ro 31-8220 (10 μM), 466 ± 50; + TPA (100 nM), 264 ± 16 d.p.m./well: mean ± S.E.M. for one representative experiment of three performed in triplicate). These results show that ET-1 can stimulate PLD activity at similar concentrations to those previously shown to cause PLC activation in this cell line [9], and that stimulation of PKC is necessary, but not sufficient, for PLD activation.

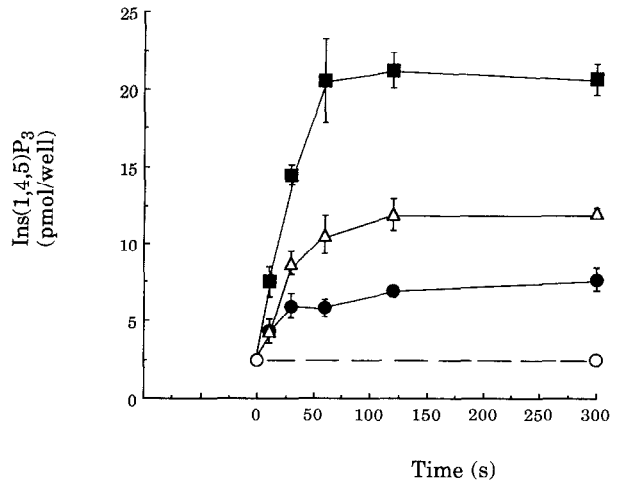

Fig. 1. Effects of Ro 31-8220 and butanol on ET-1-stimulated Ins(1,4,5)P_3 accumulation in SK-N-MC cells. Cells were pre-incubated for 10 min in the presence of 10 μM Ro 31-8220 or 50 mM butanol before addition of 100 nM ET-1. Data points shown represent means ± S.E.M for 4 separate experiments performed in triplicate. Symbols represent: ○, no addition, ●, + ET-1, ■, + ET-1/Ro 31-8220, and △, + ET-1/butanol.

Table I

Effects of Ro 31-8220 and TPA on basal and ET-1-stimulated [^{3}H]$InsP_x$ and Ins(1,4,5)P_3 accumulation in SK-N-MC cells

	No addition	Ro 31-8220 (10 μM)	TPA (100 nM)
(a) [^{3}H]$InsP_x$ accumulation (dpm/well)			
Basal	2,963 ± 222	5,054 ± 491	3,443 ± 104
+ ET-1 (100 nM)	5,389 ± 129	13,366 ± 662	3,802 ± 299
(b) Ins(1,4,5)P_3 accumulation (pmol/well)			
Basal	2.94 ± 0.33	3.95 ± 0.60	3.39 ± 0.58
+ ET-1 (100 nM)	9.09 ± 0.49	25.42 ± 2.23	3.94 ± 0.24

For (a) values are means ± S.E.M. for a single representative experiment, performed on three other occasions with similar results. SK-N-MC cells were pre-incubated with either Ro 31-8220 or TPA, plus 10 mM LiCl for 10 min before addition of ET-1 or vehicle for 30 min. For (b) values are means ± S.E.M. for at least 3 experiments performed in triplicate. Pre-incubation with either Ro 31-8220 or TPA for 10 min was followed by ET-1 challenge for 5 min. Ro 31-8220 significantly enhanced basal [^{3}H]$InsP_x$ accumulation ($P < 0.05$), but not Ins(1,4,5)P_3 accumulation. For both (a) and (b) Ro 31-8220 significantly enhanced ($P < 0.01$), and TPA significantly attenuated ($P < 0.05$) the ET-1-stimulated response (Student's t-test)

Experiments using [^{3}H]inositol-labelled cells and measurement of total [^{3}H]$InsP_x$ as an index of PLC activity (Table I) show that both basal and ET-1-stimulated [^{3}H]$InsP_x$ accumulations were increased in the presence of Ro 31-8220. TPA had no effect on basal [^{3}H]$InsP_x$, but substantially attenuated the agonist-stimulated increase in [^{3}H]$InsP_x$ accumulation. These results suggest that activation of PKC can inhibit ET-1-stimulated PLC activity, and both basal and agonist-stimulated PLC activity may be under tonic inhibitory feedback control by PKC.

These conclusions are supported and strengthened by experiments where changes in Ins(1,4,5)P_3 mass levels were measured. Time-course studies established that ET-1 (100 nM) caused a sustained 3-fold increase in Ins(1,4,5)P_3 accumulation (Fig. 1). In the presence of a maximally-effective concentration of the PKC inhibitor Ro 31-8220 (10 μM) a substantial enhancement of Ins(1,4,5)P_3 accumulation was observed (Fig. 1); thus at 5 min after ET-1 addition the increase in Ins(1,4,5)P_3 accumulation was 350% greater in the presence of Ro 31-8220 compared to the effect of ET-1 alone (Table I). In contrast to the data shown for [^{3}H]$InsP_x$ accumulation, Ro 31-8220 alone had no significant effect on basal Ins(1,4,5)P_3 levels (Table I). TPA (100 nM) had no effect on basal Ins(1,4,5)P_3 levels, but abolished the ET-1-stimulated increase in Ins(1,4,5)P_3 accumulation (Table I). These data clearly show that inhibition of PKC activity dramatically increases the Ins(1,4,5)P_3 accumulation attained in the presence of ET-1, whilst addition of an exogenous activator of PKC can essentially abolish agonist-stimulated Ins(1,4,5)P_3 accumulation,

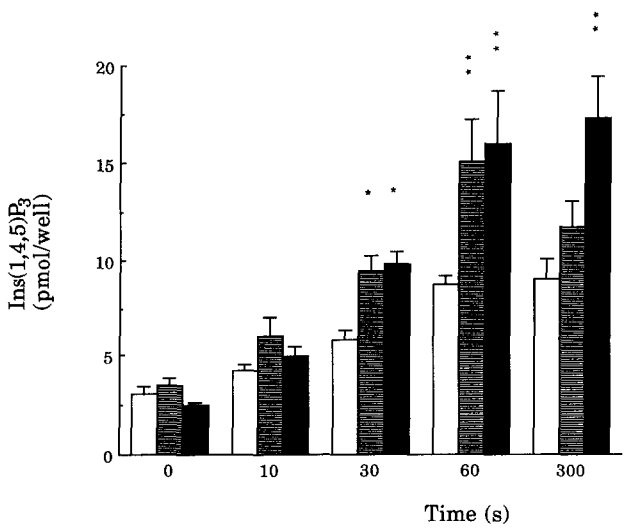

Fig. 2. Effects of DL-propranolol and butanol on ET-1-stimulated Ins(1,4,5)P_3 accumulation in SK-N-MC cells. Cells were pre-incubated for 10 min in the presence of 300 μM DL-propranolol (striped bars), 50 mM butanol (filled bars) or no pre-addition (open bars) before addition of 100 nM ET-1 at zero-time. Columns represent means ± S.E.M. for 3 separate experiments performed in triplicate. Statistically significant (Student's t-test) enhancements of the Ins(1,4,5)P_3 accumulation over those caused by ET-1 only are indicated as $*P < 0.05$; $**P < 0.01$.

establishing the existence of a short inhibitory feedback loop between PKC and PLC in SK-N-MC cells.

Further experiments to investigate the possible regulatory role of products of PLD activation were undertaken using either butanol (50 mM) to divert PLD catalysed formation of PtdOH to PtdBut, or DL-propranolol (300 μM) to inhibit PPH and thus prevent the conversion of PtdOH to diacylglycerol.

In the presence of 50 mM butanol, ET-1-stimulated Ins(1,4,5)P_3 accumulation was enhanced at all but the earliest (10 s) time-point (Figs. 1 and 2). This enhancement was characteristically 30–50% of that observed in the presence of Ro 31-8220 (Fig. 1). Butanol (50 mM) alone had no effect on basal levels of Ins(1,4,5)P_3 (Fig. 2). Preliminary experiments showed that the enhancement of the ET-1 response was concentration-dependent with respect to butanol and was maximal at a butanol concentration of 30 mM, in good agreement with the butanol concentration-dependency for maximal [^{32}P]PtdBut formation in $^{32}P_i$-loaded cells (data not shown). It should also be noted that the enhancement of ET-1-stimulated Ins(1,4,5)P_3 accumulation by Ro 31-8220 was similar in the presence and absence of butanol (ET-1/Ro 31-8220, 25.4 ± 2.2; ET-1/Ro 31-8220/butanol, 27.0 ± 2.2 pmol Ins(1,4,5)P_3/well 5 min after agonist challenge for 3 experiments performed in triplicate).

DL-Propranolol (300 μM) exerted effects on ET-1-stimulated Ins(1,4,5)P_3 accumulation which were comparable to those of butanol (Fig. 2). Each agent significantly enhanced the Ins(1,4,5)P_3 response at 30 and 60 s after ET-1 challenge. However, whereas the butanol effect was maintained over the 5 min time-course, the propranolol enhancement decreased with time.

At the concentration necessary for complete diversion of PLD activity towards PtdBut formation, butanol might be expected to have diverse effects; however, we have previously shown that where PLD is not activated as a consequence of agonist stimulation, butanol at 50 mM does not affect PLC regulation [11]. The specificity of 300 μM DL-propranolol is less certain, with the recent demonstration that this agent may also have a direct PKC inhibitory action [12]. Despite these caveats, the results of this study are consistent with the hypothesis that the effects of butanol and propranolol are to prevent PtdOH/diacylglycerol formation and thus PKC activation by products of the PLD/PPH pathway. Since butanol and propranolol would have opposing effects on PtdOH derived from PLD activity, it would appear unlikely that PtdOH (rather than diacylglycerol) can be responsible for the effects seen here. Furthermore, the lack of effect of butanol, compared to the significant enhancement of ET-1-stimulated Ins(1,4,5)P_3 accumulation caused by Ro 31-8220, at the earliest time-point studied (10 s) may reflect a slightly delayed onset of action for PKC activation by diacylglycerol derived via the PLD/PPH route. These data therefore provide novel evidence in support of the notion that PLD activation downstream of agonist-stimulated PLC can contribute to the mechanism whereby PKC can regulate PLC activity by an inhibitory feedback loop.

Many reports have demonstrated diacylglycerol formation as a consequence of PLD/PPH pathway activity following agonist stimulation. However, the ability of diacylglycerol from this source to activate PKC has been a matter of some dispute. Recently, Lin et al. [13] have reported that PLD-derived diacyl-glycerol does not initiate PKC activation, but plays an important role in maintaining PKC activation in a mast cell line. Furthermore, in the same cell line, Ozawa et al. [14] have provided evidence that both α and ε isoforms of PKC are responsible for feedback inhibition of inositol phospholipid hydrolysis. However, Pachter et al. [15] have demonstrated that over-expression of the β1 isoform of PKC in rat-6 fibroblasts increases PLD activity in the presence of thrombin, whilst dramatically attenuating PLC activation, implicating the β1 isoform as a potential mediator of the inhibitory feedback circuit. Considering these findings in the light of our own data, it is tempting to speculate that different isoforms of PKC may be involved in the inhibition of inositol phospholipid hydrolysis depending on the relative generation of diacylglycerol from PLC- and PLD-derived sources.

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